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TITLE: Predicting Disease Progression in Scleroderma with Skin and Blood Biomarkers

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14. ABSTRACT

Scleroderma (Systemic Sclerosis, SSc) is a chronic, incurable autoimmune disease associated with high morbidity and mortality primarily due to SSc-lung disease (1, 2). There is a large variability in individual patients' courses and current predictors of disease progression are inadequate. The overall objective of the proposed research is to develop reliable predictors for clinical outcomes, particularly interstitial lung disease, in scleroderma, utilizing the biospecimens and longitudinal clinical data in the GENISOS cohort to perform an analysis combining data from multiple areas to develop robust prediction models for ILD progression. The model will include genotypic data, gene expression profiling and cytokine/analyte levels, in addition to clinical parameters of pulmonary function tests and chest CAT (computer assisted tomography) scans. In the first year we have focused on patient recruitment, clinical characterization, specimen collection (DNA, RNA, skin biopsies, serum, plasma, monocytes). We have begun the analysis of serum analytes and gene expression. We have prepared 3 abstracts accepted for presentation at the annual American College of Rheumatology meeting Nov 16-19, 2014 in Boston.

15. SUBJECT TERMS

Scleroderma, Systemic Sclerosis, GENISOS (Genes versus Environment in Scleroderma Outcome Study), Interstitial Lung Disease, cytokines, DNA, RNA, skin biopsy

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Table of Contents

Page

ANNUAL PROGRESS REPORT for W81XWH-13-1-0452
for period 9/23/2013 through 9/22/201
"Predicting Disease Progression in Scleroderma with Skin and Blood Biomarkers"

1. Introduction:

Scleroderma (Systemic Sclerosis, SSc) is a chronic, incurable autoimmune disease associated with high morbidity and mortality primarily due to SSc-lung disease (1, 2). There is a large variability in individual patients' courses and current predictors of disease progression are inadequate. The overall objective of the proposed research is to develop reliable predictors for clinical outcomes, particularly interstitial lung disease, in scleroderma, utilizing the biospecimens and longitudinal clinical data in the GENISOS cohort to perform an analysis combining data from multiple areas to develop robust prediction models for ILD progression. The model will include genotypic data, gene expression profiling and cytokine/analyte levels, in addition to clinical parameters of pulmonary function tests and chest CAT (computer assisted tomography) scans. In the first year we have focused on patient recruitment, clinical characterization, specimen collection (DNA, RNA, skin biopsies, serum, plasma, monocytes). We have begun the analysis of serum analytes and gene expression. We have prepared 3 abstracts accepted for presentation at the annual American College of Rheumatology meeting Nov 16-19, 2014 in Boston. We are on track with all proposed activities.

2. Keywords

Scleroderma, Systemic Sclerosis, GENISOS (Genes versus Environment in Scleroderma Outcome Study), Interstitial Lung Disease, cytokines, DNA, RNA, skin biopsy

3. Overall Project Summary for the first year

STATEMENT OF WORK from original application followed by responses. All Tasks that were to be done within the first year are highlighted.

Task 1: **Institutional Review Board (IRB) and DOD Human Research Protection Office (HRPO)**

1.a. Local IRB Approval (months 1-2)

"Skin and blood samples are currently collected in the GENISOS cohort based on the existing protocols approved by the UTHealth (Houston, TX) IRB. We will modify the study protocol based on the proposed research and obtain approval from the local IRB at UTHealth."

1.b. DOD HRPO Approval (month 3)

"The study protocols and consent forms approved by the local IRB will be submitted to DOD HRPO for review and approval."

ANNUAL PROGRESS REPORT RESPONSES - Current Objectives, Results, Progress and Accomplishments, Discussion: the initial approval has been completed; annual reviews are up-to-date and will be ongoing throughout the study. The HRPO has been kept informed of the annual reviews and approvals.

Task 2: **Collection of DNA samples, genotyping and analysis of genetic data (Specific Aim 1)**

2.a. Collection of DNA samples (months 1-36) "Forty new patients will be enrolled annually by Drs. Mayes and Assassi into the GENISOS cohort at UTHealth. DNA will be extracted from baseline blood samples by the Research Assistant in the laboratories of Division of Rheumatology at UTHealth."

ANNUAL PROGRESS REPORT RESPONSES - Current Objectives, Results, Progress and Accomplishments, Discussion: Forty new patients have been enrolled in the past year; baseline blood samples have been collected – see Tables 1 (proposed sample

collection schedule) and Table 2 (actual sample collection schedule). We are on target with this.

2.b. Genotyping by Taqman Assay (months 25-30)

"Genotyping data are available on the majority of patients in the GENISOS cohort through genome wide association study and Immunochip efforts. Genotyping by Taqman assays will be performed for selected susceptibility loci in the newly enrolled patients (120 by the end of funding period). The assays will be completed by the Research Assistant under Dr. Mayes's supervision in the laboratories of Division of Rheumatology at UTHealth."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness.

2.c Analysis of genetic data (months 31-32)

"The predictive significance of selected susceptibility loci for disease severity will be examined. The analysis will be performed by Drs. Pedroza (UTHealth) and Gorlova (UT M.D. Anderson Cancer Center, Houston, TX)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness.

2.d. Manuscript on genetic predictors of disease progression (months 33-34)

"A manuscript on genetic predictors of disease progression will be prepared by Drs. Mayes and Assassi (UTHealth) as well as Dr. Gorlova (UT M.D. Anderson Cancer Center)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness.

Task 3: Collection of skin samples, RNA extraction, gene expression analysis (Specific Aim 2)

3.a. Collection of skin biopsy samples (months 1-24)

"Skin biopsy samples will be collected from newly enrolled patients and a subgroup of patients seen at year 1 visit. 80 skin biopsy samples have been already collected. Drs. Mayes and Assassi will perform 60 skin biopsies per year at UTHealth. The skin biopsy samples are stored in RNAlater in -80 freezer. The sample collection and study design will be conducted in consultation with Dr. Michael Whitfield at Dartmouth Medical School (Dartmouth, NH)."

Current Objectives, Results, Progress and Accomplishments, Discussion: Skin biopsy samples have been collected from 96 subjects (39 at baseline and 56 at follow-up visits - see Table 2 below). All samples have been processed per protocol and stored at -80 freezer in RNAlater solution. Conversations with Dr. Whitfield have been ongoing throughout the year – both electronically and in person at the November 2013 American College of Rheumatology (ACR) annual meeting. A meeting is also planned for the November 2014 (Nov 16-19, 2014)ACR annual meeting in Boston. We are on track for this task.

3.b. RNA extraction and global gene expression study in skin samples (months 25-26)

"Purified RNA will be extracted from stored skin biopsy samples by the Research Assistant using commercially available kits in the laboratories of Division of Rheumatology at UTHealth. The RNA quantity and quality will be assessed by Nanodrop and Bionanalyzer in the CTSA Microarray Core Laboratories at UTHealth. Global gene expression profiling will also be performed in the Microrray Core Laboratories."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness.

3.c. Analysis of skin global gene expression data (months 27-30)

"The analysis of skin gene expression data will be completed by Drs. Assassi (UTHealth) and Gorlova (UT M.D. Anderson Cancer Center) in consultation with Dr. Michael Whitfield at Dartmouth Medical School (Dartmouth, NH)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness.

3.d. Manuscript on skin gene expression predictors of disease progression (months 30-32)

"A manuscript on skin gene expression predictors of disease progression will be prepared by Drs. Mayes and Assassi (UTHealth)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness.

Task 4: Collection of monocyte samples, RNA extraction, gene expression analysis (Specific Aim 2)

4.a. Collection of monocyte samples (months 1-24)

"Blood samples will be collected in CPT tubes from newly enrolled patients and patients seen at year 1 visit. 80 monocyte samples have been already collected. Drs. Mayes and Assassi will conduct the GENISOS visit and collect the clinical data. The monocyte samples will be purified and the RNA will be extracted by the Research Assistant in the laboratories of Division of Rheumatology at UTHealth. The purified RNA samples will be stored in -80 freezer."

Current Objectives, Results, Progress and Accomplishments, Discussion: Blood samples have been collected from 42 out of 43 newly enrolled samples. Not all subjects provided a blood sample. Follow-up samples have been collected from 121 subjects, as noted in Table 2. Monocytes have been purified, RNA extracted and stored per protocol. Although the number of blood samples from newly enrolled subjects is not quite up to the numbers proposed, we have "over-sampled" individuals for follow-up and we will be able to compare changes over time.

4.b. Global gene expression study in monocyte samples (month 24)

"The RNA quantity and quality will be assessed by Nanodrop and Bionanalyzer in the CTSA Microarray Core Laboratories at UTHealth. Global gene expression profiling will also be performed in the Micrrray Core Laboratories."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

4.c. Analysis of monocyte global gene expression data (months 25-28)

"The analysis of mococyte gene expression data will be completed by Drs. Assassi (UTHealth) and Gorlova (UT M.D. Anderson Cancer Center)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

4.d. Manuscript on monocyte gene expression predictors of disease progression (months 29-30)

"A manuscript on monocyte gene expression predictors of disease progression will be prepared by Drs. Mayes and Assassi (UTHealth)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

Task 5: Cytokine level determination and data analysis (Specific Aim 3)

5.a. Multiplex assays in rapid/slow progressor groups (months 6-10)

"Human DiscoveryMAP v 1.0 multiplex assays for the analyte determination in already collected 62 samples (rapid and slow progressor samples-Stage I of Specific Aim 3) will be performed by Myriad Ruled Based Medicine (Austin, TX)."

Current Objectives, Results, Progress and Accomplishments, Discussion: We have performed cytokine analysis on 10 cases and 10 controls, using a multiplex system that measured 45 analytes including cytokines, chemokines and acute-phase reactants. Results indicated that six of these analytes were differentially expressed between cases and controls and correlated with microRNA profiles. We are at the beginning of these experiments and analysis but this clearly has implications for predicting disease activity. Our next step will be to determine these levels in larger numbers of subjects (on samples already collected or being collected) and to measure changes over time. A report of these results will be presented at the annual American College of Rheumatology (ACR) meeting in November, 2014 in Boston.

5.b. Analysis data of Human DiscoveryMAP multiplex assays (months 10-16)

"The data from Human DiscoveryMAP multiplex assays will be conducted by Dr. Pedroza at UTHealth. A limited number of cytokines will be identified that differentiate the fast progressor group from slow progressors."

Current Objectives, Results, Progress and Accomplishments, Discussion: This analysis has been done on the first 10 subjects and will be expanded as noted in the paragraph above.

5.c. Design of custom-made multiplex assay (months 16-20)

"Custom-made multiplex assays will be designed in collaboration with Myriad Ruled Based Medicine (Austin, TX) based on the identified cytokines in the Subtask 5.b."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

5.d. Determination of cytokine levels by custom-made assays (months 23-24)

The levels of selected cytokines will be determined in all collected baseline samples (currently available 331 + newly enrolled 80= 411) utilizing custom-made assays. These assays will be performed by Myriad Ruled Based Medicine (Austin, TX).

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

5.e. Manuscript based on the cytokine data (months 25-27)

"The predictive significance of determined cytokine levels in Subtask 5.d. will be examined for disease progression. This analysis will be performed by Dr. Pedroza (UTHealth). A manuscript will be prepared based on these results by Drs. Mayes, Assassi, and Pedroza (UTHealth)"

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

Task 6: Multivariable models with identified clinical and molecular predictors (Specific Aim 4)

6.a. Random forest and longitudinal analyses (months 32-34)

'The identified molecular predictors in Tasks 2-5 will be analyzed along with clinical predictors. Random forest analysis will be used for data reduction. Multivariable joint analysis of

longitudinal measurements and survival data will be performed. These analyses will be performed by Drs. Pedroza (UTHealth) and Gorlova (UT M.D. Anderson Cancer Center)."

Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness

6.b Manuscript on multivariable models predictive of disease progression (months 35-36)

"A manuscript reporting on multivariable predictors of disease progression will be prepared by Drs. Mayes, Assassi, Pedroza (UThealth) and Dr Gorlova (UT M.D. Anderson Cancer Center)." ***Not applicable to the first year (months 1-12); included here for the sake of transparency and completeness***

Task 7: Calculation of fibrosis score of high resolution chest CTs (months 0-36)

"Dr. Ferguson will determine the fibrosis score on the high resolution chest CTs obtained in the GENISOS cohort"

Current Objectives, Results, Progress and Accomplishments, Discussion: Dr. Ferguson has scored 75 chest CTs on 59 patients over the course of this year. In addition she has presented these data to our monthly scleroderma research conference. This is an integral part of our outcome analysis. We are on track with this.

Task 8: Expansion of the GENISOS cohort (months 0-36)

"The baseline and follow-up GENISOS visits will be conducted by Drs. Mayes and Assassi at UTHealth. This is necessary for capturing the longitudinal clinical data and collecting the biospecimens for the proposed research."

Current Objectives, Results, Progress and Accomplishments, Discussion: We have reached, and actually exceeded, our goal of 40 newly recruited subjects as we have enrolled a total of 43 cases. All clinical data and biospecimens have been collected per protocol (see Tables 1 and 2 below).

Task 9: Maintenance and expansion of GENISOS data base (months 0-36)

"The Data Base Manager at UTHealth in close collaboration with Mr. Tony Mattar (Computer Task Force, Inc, Troy, MI) will maintain the GENISOS data base. The data base will also be expanded to accommodate the genetic and cytokine data. They also will establish an interface for connecting the gene expression data with the clinical data base."

Current Objectives, Results, Progress and Accomplishments, Discussion: tracking of all skin biopsies and blood samples has been added to the database, all visit data has been checked for quality (through our data quality measures) and entered.

Table 1. PROPOSED Quarterly and Cumulative Sample Collection Schedule for year 1:

Time/ Months	Newly Enrolled/ f/u[1] visits	Skin Baseline	Skin f/u	Monocyte Baseline	Monocyte F/U	DNA Baseline	Serum/Pax[2] Baseline	Serum/Pax f/u
0-3	10/20	10	5	10	5	10	10	22
4-6	10/20	10	5	10	5	10	10	22
7-9	10/20	10	5	10	5	10	10	22
10-12	10/20	10	5	10	5	10	10	22
Cumula-tive: 0-12	40/80	40	20	40	20	40	40	88

[1] Number of newly enrolled subjects/ follow-up visits

[2] Pax = pax gene tubes for RNA collection

Table 2. ACTUAL QUARTERLY AND CUMULATIVE (to date) SAMPLE COLLECTION for year 1:

Time/ Months	Newly Enrolled/ F/U visits	Skin Baseline	Skin F/U	Monocyte Baseline	Monocyte F/U	DNA Baseline	Serum/Pax Baseline	Serum/Pax F/U
0-3	10/18	10	6	10	10	10	10 serum/ 10 Pax	10 serum/ 10 Pax
4-6	11/37	9	8	11	11	9	9 serum/ 9 Pax	11 serum/ 11 Pax
7-9	13/20	10	17	13	20	12	12 serum/ 13 Pax	25 serum/ 19 Pax
10-12	10/46	10	26	10	46	10	10 serum/ 10 Pax	47 serum/ 46 Pax
Cumula-tive: 0-12	43/121	39	57	44	87	41	41 serum/ 42 Pax	93 serum/ 86 Pax

F/U = follow-up
Pax = Pax gene collection for RNA

4. Key Research Accomplishments

a. The collection of the largest longitudinal sample repository in scleroderma that will permit completion of all proposed experiments.
b. Results of our cytokine analysis (reported in abstract #1 listed below entitled "The Global miRNA Whole Blood Profile in Systemic Sclerosis and Its Correlation with Serum Cytokine Levels") suggest a link of miRNA to the pathogenesis of SSc and could have important ramifications for future drug and biomarker development
c. The analysis of our gene expression data (reported in abstract #2 listed below entitled "Dissecting the Heterogeneity of Skin Gene Expression Patterns in Systemic Sclerosis") showed a prominent keratin signature in addition to the fibro-inflammatory signature indicating that the dysregulation in SSc skin is not confined to the dermis (as currently believed) but also involves other cell compartments. This is a novel finding and potentially useful for stratifying patients for interventions.
d. Analysis of genetic risk factors for interstitial lung disease in the GENISOS cohort (reported in abstract # 3 listed below entitled "Genetic Susceptibility Loci of Idiopathic Interstitial Pneumonitis do not Represent Risk for Systemic Sclerosis") indicating that these 2 conditions, although phenotypically similar, have quite distinct genetic risk factors.

5. Conclusions

In summary, this first year has seen the organization of the study, ongoing recruitment and expansion of our cohort and the collection and processing of multiple samples as proposed in our original application and summarized above. We have begun the process of measuring cytokines/analytes and interpreting these levels in light of disease progression.

Future plans will include continued recruitment, follow-up, data and sample collection from the GENISOS cohort. The second year will be devoted to the analysis of changes over time in these parameters with emphasis on predictors of disease progression (or lack thereof).

6. Publications, Abstracts, and Presentations

a. Manuscripts:

a.1.Lay Press: Nothing to report.

a.2.Peer-Reviewed Scientific Journals:

1. Wiese AB, Berrocal VJ, Furst DE, Seibold JR, Merkel PA, **Mayes MD**, Khanna D. Correlates and responsiveness to change of measures of skin and musculoskeletal disease in early diffuse systemic sclerosis. Arthritis Care Res (Hoboken). 2014 Apr 1. doi: 10.1002/acr.22339. [Epub ahead of print] PMID:24692361

a.3.Invited Articles:

1. Wu, M, **Mayes MD**. Insights into the genetic basis of systemic sclerosis: Immunity in human disease and SSc mouse models. Adv Genomics Genet. 2014 *In press*.

a.4. Abstracts:

1. Salazar GA, Hagan J, Wu M, **Mayes MD**, Reveille HD and Assassi S. The Global miRNA Whole Blood Profile in Systemic Sclerosis and Its Correlation with Serum Cytokine Levels. Accepted for poster presentation at American College of Rheumatology Annual Meeting, 11/2014.
2. Assassi, S, Swindell WR, Wu M, Tan FK, Khanna D, Furst DEF, Tashkin DP, Jahan-Tigh RR, **Mayes MD**, Gudjonsson JE, Chang JT. Dissecting the Heterogeneity of Skin Gene Expression Patterns in Systemic Sclerosis. Accepted for poster presentation at American College of Rheumatology Annual Meeting, 11/2014.
3. Wu M, Assassi S, Salazar GA, Gorlova OY, Chen W, Charles J, Wigley F, Hummers L, Varga J, Hinchcliff M, Khanna D, Schiopu E, Phillips K, Furst DE, Steen V, Baron M, Hudson M, Taillefer SS, Pope J, Jones N, Markland J, Docherty P, Khalidi NA, Robinson D, Simms R, Silver R, Frech TM, Fessler B, Molitor J, Fritzler M, Segal B, Al-Kassab F, Yang J, **Mayes MD**. Genetic Susceptibility Loci of Idiopathic Interstitial Pneumonitis do not Represent Risk for systemic sclerosis. Accepted for poster presentation at American College of Rheumatology Annual Meeting, 11/2014.

2. Inventions, Patents and Licenses – Nothing to report

3. Reportable Outcomes – Scientific Advances:

a. Publication of the first microRNA whole blood profiling in SSc as described in Abstract #1 above.
b. The discovery of the keratin signature in SSc skin described in Abstract # 2 above.
c. The discovery that the susceptibility genes of idiopathic interstitial lung disease are quite distinct from those of SSc-related ILD.

4. Other Achievements

A repository of DNA, RNA**,** serum and plasma has resulted from the sample collection for this study. This repository is linked to longitudinal clinical data for ready analysis of predictors of outcome.

5. References

1. Elhai M, Meune C, Avouac J, Kahan A, Allanore Y: Trends in mortality in patients with systemic sclerosis over 40 years: a systematic review and meta-analysis of cohort studies. *Rheumatology (Oxford)* 2011.
2. Thomas E, Symmons DP, Brewster DH, Black RJ, Macfarlane GJ: National study of cause-specific mortality in rheumatoid arthritis, juvenile chronic arthritis, and other rheumatic conditions: a 20 year followup study. *J Rheumatol* 2003, 30: 958-965.

6. Appendices – Nothing to report